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An improved enrichment method for functionally competent, highly purified peripheral blood dendritic cells and its application to HIV-infected blood samples

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SUMMARY

Dendritic cells (DC) were purified from human peripheral blood using a rapid and simple method based on magnetic depletion of phagocytes with carbonyl iron, followed by centrifugation of non-phagocytic cells on a Percoll density gradient and depletion of lymphocytes and macrophages/monocytes with a panel of MoAbs and immunomagnetic beads. Enriched DC were obtained with >99% purity as judged by non-specific esterase (NSE) staining. After isolation, these cells, representing 0·4% of the starting mononuclear cell population, still function as potent antigen-presenting cells for purified T lymphocytes. The present results confirm the ability of human peripheral blood DC to present soluble antigens to T cells including microbial antigens and show, further, that DC are more potent soluble antigen-presenting cells than monocytes. The method was successfully applied to the purification of DC from the blood of HIV-infected individuals. We could not detect decreased numbers of DC in four individuals with early HIV infection and no replicating HIV was detected by *in situ* hybridization in the DC.

Keywords dendritic cells antigen presentation HIV

INTRODUCTION

Dendritic cells (DC) have been described in lymphoid tissues of many species, and related cells are present in most non-lymphoid compartments of the body. DC are Ia-positive [1–3] but lack surface immunoglobulin, Fc receptors, the receptor for sheep erythrocytes, and several lymphocyte and monocyte surface antigens detectable by MoAbs [4–8]. Functional studies have indicated that DC are potent, non-phagocytic stimulator cells in both allogeneic and autologous mixed lymphocyte reactions [5,9–12] and as antigen-presenting cells (APC). It is widely accepted that both *in vivo* and *in vitro* T cell antigen responses are initiated by interaction with APC expressing MHC class II antigens and presenting the antigen in context with MHC. MHC molecules are not necessary for T cell response to mitogenic stimuli [13,14]. The major APC are phagocytic macrophages [15–17] and non-phagocytic dendritic cells [4,10]. Although DC are scant in the peripheral blood of man, they are potent inducers of T cell proliferation *in vitro* [5,11,18]. Murine DC have also been shown to induce primary antiviral proliferative T cell responses and to generate virus-specific cytotoxic T cells (CTL; [19]).

The enrichment of DC has been difficult because these cells are present in many tissues with a frequency of less than 1% [8]. The techniques used to purify DC from peripheral blood mononuclear cells (PBMC) have been based on the semiadherent behaviour of these cells and the fact that they localize to the interface on Ficoll gradient. However, the cell population so separated contains, in addition to DC, a large number of monocytes which have been difficult to remove because of their low density [8]. Although MoAbs, directed against monocyte-, T, and B lymphocyte-specific surface molecules [6], have made the purification of the respective cells possible, MoAbs to human peripheral blood DC-specific surface determinants are not yet available. Thus, it has been considered that the distinct morphology of DC seen with the May–Grünwald–Giemsa (MGG) staining may be the only positive criterion for these cells to date [20].

The present paper describes a rapid and simple technique for purifying DC from human peripheral blood. In addition to adherence our method uses magnetic depletion of phagocytes

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with carbonyl iron to obtain a preparation essentially free of contaminating macrophages. After centrifugation of non-phagocytic cells on a Percoll density gradient, contaminating T and B lymphocytes, natural killer (NK) cells, and monocyte/macrophages are eliminated by treatment with a panel of MoAbs and immunomagnetic beads. DC thus purified are shown to be functionally active and to surpass the antigen-presenting capacity of purified monocytes. Our studies were designed also to enrich DC from the blood of HIV-infected patients to make studies possible on the eventual infection and/or loss of function of DC in early HIV infection.

MATERIALS AND METHODS

Human blood samples

Altogether 32 buffy coats obtained from healthy blood donors (Red Cross Transfusion Service, Tampere, Finland, and Department of Transfusion Medicine, National Institutes of Health, Bethesda, MD) served as the source of DC in the following experiments. For the studies of DC in early HIV infection, peripheral blood (60 ml) was obtained, after informed consent, from four HIV-infected patients who had contracted HIV infection 0·5–2 years earlier. Clinically, the patients presented with CDC stage II or III disease. The patients were seen at the Department of Dermatology and Venereology, Helsinki University Central Hospital, and the study was approved by the hospital's ethical committee.

Isolation of peripheral blood mononuclear cells

Mononuclear cells were isolated from fresh citrated buffy coats by density centrifugation on Ficoll–Isopaque gradient (Pharmacia Ltd., Uppsala, Sweden; [21]). The interface layer, enriched in lymphocytes, monocytes/macrophages and dendritic cells, was washed three times with complete medium containing RPMI 1640 supplemented with 10% fetal calf serum (FCS; GIBCO, Grand Island, NY), and resuspended to a concentration of 5×10^6 cells/ml. To isolate different subpopulations of PBMC, 15–30 ml of the cell suspension were dispensed on 30 mm plastic Petri dishes (Nunc-Intermed, Roskilde, Denmark) and incubated for 2 h at 37°C in a humidified atmosphere of 5% CO_2 in air. Subsequently, the non-adherent cells were removed with 6–8 rinses of complete medium and used as a source of T lymphocytes and dendritic cells.

Preparation of purified dendritic cells

Non-adherent cells were collected and incubated for 1 h with carbonyl iron (Sigma, St Louis, MO), 1 mg/10^6 cells in complete medium at 37°C with mild agitation. Free iron and cells which had phagocytized the iron were removed with a magnet (Dynal Corp., Oslo, Norway). A maximum of 50×10^6 cells of the recovered non-phagocytic cell population were placed on 5 ml of 50% Percoll (Pharmacia) and centrifuged at 300 *g* for 30 min at room temperature. The interface cells were collected and washed by pelleting three times. All washes were made with complete medium during the preparation of DC.

The cell concentration was adjusted to $10–20 \times 10^6$/ml. This cell suspension was incubated with the following MoAbs (Becton Dickinson, Burlingame, CA): anti-Leu-M1 (monocytes; CD15), anti-Leu-M3 (macrophages, mature monocytes), anti-Leu-12 (B cells; CD19), anti-Leu-11B (NK cells; CD16), anti-Leu-3a + anti-Leu-3b (T helper/inducer cells; CD4) and anti-Leu-4 (T cells; CD3). Ten microlitres of each MoAb were used for each 1 ml cell suspension and samples were placed on a rotator at 4°C for 30 min. The cells were then washed twice with complete medium.

Immunomagnetic beads (IB) (Dynal Inc., Fort Lee, NJ), coated with goat anti-mouse IgG, were washed five times in complete medium using a magnet. The washed beads were added to the above cell solution at 7·5 ml/10^6 cells and incubated at 4°C for 2 h for negative selection. Beads were removed with a magnet and the cells were washed twice. Cell samples were taken at different steps of the isolation procedure to check for purity of the obtained cells.

Preparation of purified monocytes

Strongly adherent cells were isolated on the basis of their receptor-mediated attachment to fibronectin on gelatin-coated surfaces [22,23]. Briefly, PBMC (2×10^6/ml) in complete medium were placed on Petri dishes (Falcon, Oxnard, CA) coated with gelatin (Sigma) and then preincubated with autologous heparinized plasma for 30 min at room temperature followed by 60 min at + 4°C for binding fibronectin to collagen. Excess plasma was removed and the plates were washed twice with cold RPMI. After incubation for 60 min at room temperature non-adherent cells were removed. Petri dishes were then incubated with RPMI 1640 containing 3·5 mM ethylene diaminotetra-acetic acid (EDTA) for 10 min at 4°C to detach the adherent cells. The purity of monocytes was determined with non-specific esterase (NSE) staining [24].

Preparation of purified T lymphocytes

Human T lymphocytes were enriched by neuraminidase-treated (*Vibrio cholerae* neuraminidase, Behringwerke AG, Marburg, Germany) sheep erythrocyte rosetting. In brief, 10^7 lymphocytes in 0·25 ml of RPMI 1640, 0·25 ml of FCS and 0·5 ml of 10^5 neuraminidase-treated sheep erythrocytes were mixed, pelleted at 1200 *g* and incubated on ice for 30 min, followed by centrifugation on a Ficoll–Isopaque gradient. Contaminating sheep erythrocytes were lysed by a hypotonic shock with distilled water. The purity of the resulting T cell population was determined with fluoresceinated MoAbs (anti-Leu-4 to T lymphocytes, CD3 and anti-Leu-16 to B lymphocytes, CD20, Becton Dickinson, Erembodegem-Aalst, Belgium), and fluorescence microscopy.

Assay of antigen-presenting function

The ability of APC to stimulate T cell proliferation was assessed in autologous cell combinations. Cultures were set up with either 10^5 PBMC or 10^5 enriched T cells with or without 0·5, 1 or 2×10^3 DC, or 1, 2 or 5×10^4 monocytes in round-bottomed microtitre plates (Nunc-Intermed, Roskilde, Denmark) in RPMI 1640 supplemented with 10% autologous plasma. The cells were cultured with or without mitogen or antigen for 3 (mitogen) or 6 (antigen) days at 37°C. Cultures without stimulus or APC alone served as controls. Cell proliferation was measured with ^{3}H-thymidine (Amersham International, Amersham, UK) incorporation and the radioactivity was measured in LKB Rackbeta II 1215 Scintillation Counter using toluene-based scintillation fluid. Quadruplicate or triplicate cultures were set up as follows: (i) PBMC with or without antigen/mitogen; (ii) purified T cells with or without antigen/mitogen; (iii) purified T cells with purified DC or monocytes with or

without antigen/mitogen; (iv) DC or monocytes alone with or without antigen/mitogen. Stimulation index (SI) was determined as the ratio of incorporation of ^{3}H-thymidine in the stimulated lymphocyte cultures (mean of quadruplicate/triplicate) divided by that of the unstimulated lymphocyte cultures.

Proliferative responses were measured to 5 μg/ml of concanavalin A mitogen (Con A, Pharmacia), to 10 μg/ml of soluble tuberculin antigen (PPD, purified protein derivative of tuberculin (State Serum Institute, Denmark)), to 1:40 dilution of soluble *Candida albicans*, referred to as CAs (ALK, Allergologisk Laboratorium A/S, Copenhagen, Denmark) and to 1:100 ratio of heat-killed particulate *C. albicans*, referred to as CAp.

Morphologic studies

For light microscopy, cytocentrifuge (Shandon, Elliot cytospin) preparations were fixed in methanol and stained with MGG. NSE staining with α-naphthyl acetate as substrate [24] was used to differentiate NSE-negative DC from NSE-positive monocytes and macrophages and to determine the purity of monocytes. For electron microscopy, purified DC were fixed initially in 2% glutaraldehyde (EM grade, Polysciences, Warrington, PA) in cacodylate buffer pH 7·4. Post-fixation in 1% osmium tetroxide was followed by *en bloc* staining in 2% aqueous uranyl acetate. After ethanol dehydration cells were embedded in Spurr's resin (Polysciences). Thin sections were stained with lead citrate, examined and photographed in a Jeol 100B electron microscope operating at 80 kV.

In situ *hybridization for HIV-specific RNA*

To demonstrate HIV-specific RNA in the isolated cells, the cells were cytocentrifuged on precleaned slides and fixed with 4% paraformaldehyde for 1 min and thereafter stored in 70% DEPC-EtOH solution. *In situ* RNA hybridization was then performed on the slides using ^{35}S-labelled RNA probes specific for HIV-1 *gag-pol* and *env* sequences as previously described [25,26]. To visualize the hybridized product as silver grains, the slides were autoradiographed. In each hybridization experiment, both non-infected and HIV-infected H9 cells were used as negative and positive controls. *In situ* RNA hybridization for HIV-specific sequences is routinely used at the Department of Dermatology, Helsinki University Central Hospital to demonstrate HIV mRNA in blood cells and other tissues.

RESULTS

Purity of cell fractions and morphology of dendritic cells

After the first step of DC purification, centrifugation on Ficoll-Isopaque, 60% of the interface cells were NSE-negative and had short cytoplasmic processes, which were also seen after MGG-staining. After removing the iron-phagocytic cells, 90% of the cells were NSE-negative. The final cell preparation from the Percoll gradient interface after treatment with MoAb and IB showed >99% NSE-negative cells (Table 1). The average yield of DC in 32 experiments was 0·4%, calculated from the Ficoll-Isopaque-purified mononuclear cells (Table 1). Light microscopic study from MGG-stained preparations showed variably shaped, roundish cells, which had veiled processes some of which were particularly long. Small cytoplasmic vacuoles and lightly basophilic cytoplasm were noted (Fig. 1a). Transmission electron micrographs (Fig. 1b) showed the remaining cells to be morphologically consistent with DC. The nuclei of these cells were large and irregular in shape, containing dense heterochromatin lining of the nuclear membrane. The nucleolus was large and centrally located. The cytoplasm contained mitochondria, large Golgi apparatus, little rough endoplasmic reticulum and no granules. Lysosomes and some vacuoles were occasionally present in the cytoplasm. The cell surface showed varying numbers of dendritic projections.

Table 1. Separation of dendritic cells from human peripheral blood*

Cell fraction and treatment	NSE-negative cells (%)	Cell yield (per cent of starting population)
Buffy coat		
Ficoll-Isopaque	ND	ND
Mononuclear cells	60	100
Carbonyl iron		
Non-phagocytic cells	90	56
50% Percoll		
Low-density cells	95	2·9
Monoclonal antibodies		
Immunomagnetic beads		
Enriched dendritic cells	100	0·4

* Figures are mean values of 32 experiments in which the s.d. was always less than 10%.

ND, Not determined; NSE, non-specific esterase.

The role of APC in mitogen/antigen-induced T cell proliferation

To demonstrate the functional capacity of the enriched DC, they were cocultured with purified T lymphocytes. For comparison, purified monocytes were also cocultured with T lymphocytes. The T cell population, purified and enriched with neuraminidase-treated sheep erythrocytes, consisted of 95–99% Leu-4$^+$ cells, <1% B cells and occasional monocytes. Cell viability was >90% by Trypan blue exclusion. The enriched monocytes were >92% NSE-positive and their viability always exceeded 95%.

Purified T cells showed a dose-dependent increase in the proliferative response to all antigens upon the addition of monocytes. In the absence of monocytes, T cells were found to be unresponsive to antigens. Thus, T cell populations were not significantly contaminated with APC. The result of one typical experiment (of three) is illustrated in Fig. 2a.

At all tested concentrations, DC effectively presented soluble and particulate antigens (Fig. 2b and 3a). However, soluble *Candida* antigen was more efficiently presented than the corresponding particulate antigen. In contrast, monocytes presented particulate *Candida* antigen more efficiently than DC (Fig. 2a). Both DC and monocytes failed to induce antigen-dependent T cell responses to levels obtained with unfractionated PBMC except for soluble *Candida* antigen (Fig. 3b).

Peripheral blood DC in HIV infection

Table 2 illustrates the yield of enriched DC from peripheral blood of four HIV-infected individuals. The average yield of DC was 1·47% (range 0·3–3·4%) of starting mononuclear cells. The blood samples were obtained 0·5–2 years after contracting HIV infection and before the loss of CD4 lymphocytes. When RNA *in situ* hybridization was performed on the DC thus purified,

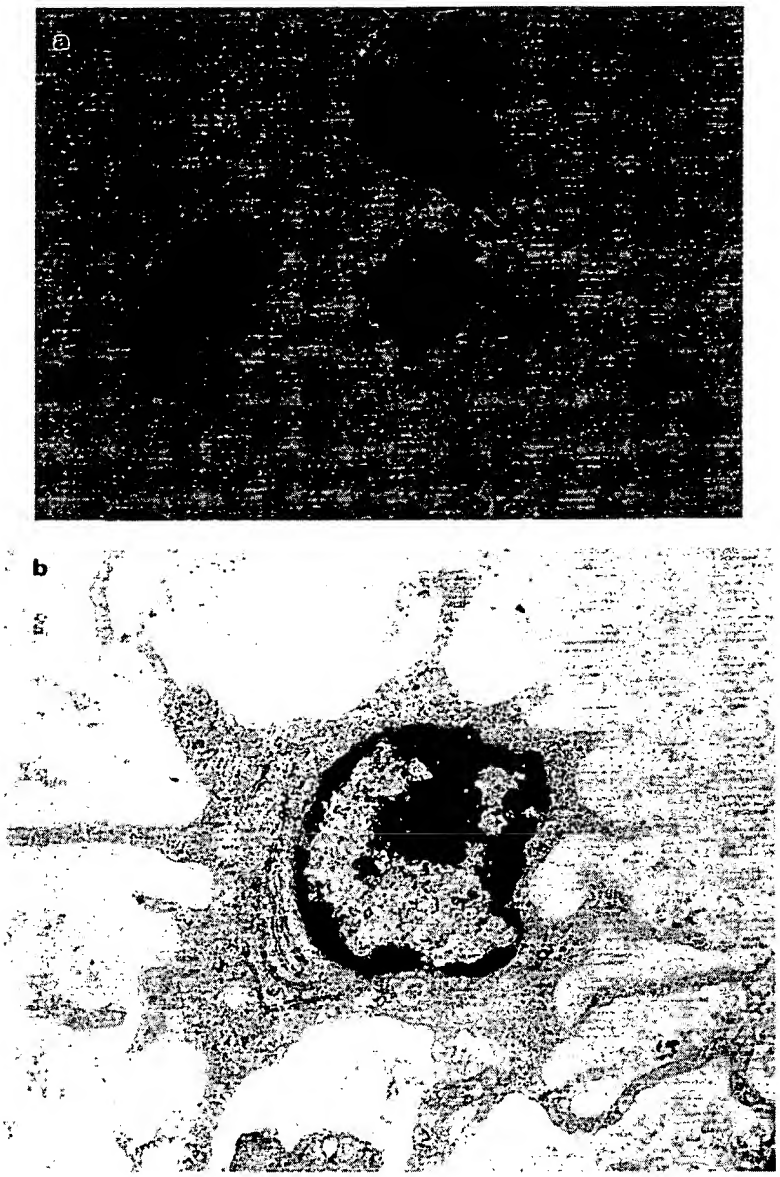

Fig. 1. (a) Light photomicrograph of the purified dendritic cells (DC). In two cells long, delicate dendritic processes are seen (× 1000). (b) Transmission electron micrograph showing a mature, activated dendritic cell with long veiled processes. The nucleus is irregularly shaped and contains heterochromatin. Endoplasmic reticulum and mainly peripherally located vacuoles are seen in the cytoplasm (× 11 000).

positive signals were detected neither for HIV *gag-pol* nor for HIV *env* mRNA. According to our experience, less than 0·1% of Ficoll-purified, non-stimulated mononuclear cells express HIV mRNA (Ranki, unpublished observation).

DISCUSSION

In this study, we first describe a rapid and simple method for the enrichment of DC from Ficoll-separated human PBMC and demonstrate the functional capacity of DC purified in this way. The purity of DC (non-adherent, non-phagocytic, NSE-negative cells) obtained with this method was close to 100%. Peripheral DC, which express no known specific surface marker, are present in human blood in trace numbers [27] and their isolation has been difficult. Our method does not require overnight incubation or complicated equipment. The yield and purity of our DC preparations are at least as good as those obtained by other purification protocols [6,20,28] and better than some others [8,29]. However, our method might not capture DC at later stages of differentiation with phagocytic capability, and associated with antigen processing in tissues. Also, as interferon-gamma (IFN-γ) may lead to the expression

of CD4 molecules on DC, our method may have removed any such cells.

It has been demonstrated that DC derived from human peripheral blood act as potent inducers of *in vitro* T cell proliferative responses to T cell antigens [5,18]. The present enrichment method does not disturb the functional capability of the DC and our results confirm the ability of human DC to present soluble antigens to T cells [30].

It has been suggested that DC are unable to present insolubilized antigens, possibly because the cells are not capable of endocytosing antigens of this kind. However, DC can present particulate antigens in collaboration with macrophages [31]. This phenomenon can explain our findings of low T cell response to particulate CA even when 2% DC were added, supporting other data on the purity of the DC population used. Similarly, the response of DC to antigenic stimulation in the absence of T cells was as low as that of T cells without the addition of APC (< 150 ct/min), confirming the purity of our DC population.

It has been postulated that DC are superior to mononuclear phagocytes in antigen presentation [27,30,32]. Our results, however, confirmed this for the soluble *Candida* antigen only. In the case of the presentation of particulate antigen, monocytes were more efficient than DC.

As peripheral blood DC can be infected by HIV *in vitro* [33], and as infection of antigen-presenting dendritic cells may play a pivotal role in the early immune deficiency of HIV infection [25,34–36], we wanted to determine whether the described

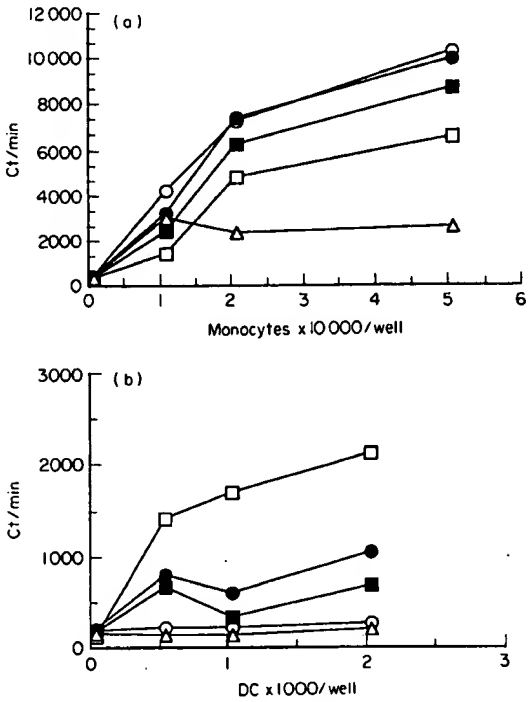

Fig. 2. The effects of increasing numbers of antigen-presenting cells (APC) on T lymphocyte proliferative responses to antigens and mitogen. Representative results of one of three experiments are shown. T lymphocytes (10^5) were cultured with graded numbers of monocytes (a) or dendritic cells (DC) (b) in the presence of concanavalin A (Con A), (5 μg/ml), soluble *Candida albicans* (CAs), (1:40), particulate CA, CAp (1:100) and purified protein derivative (PPD) (10 μg/ml). In absence of T cells, the ct/min values of monocytes/DC control cultures were < 500/150 respectively. The results are expressed as mean ct/min values of triplicate cultures, with s.d. always < 20%. ○, Con A; ●, PPD; ■, CAp; □, CAs; △, control.

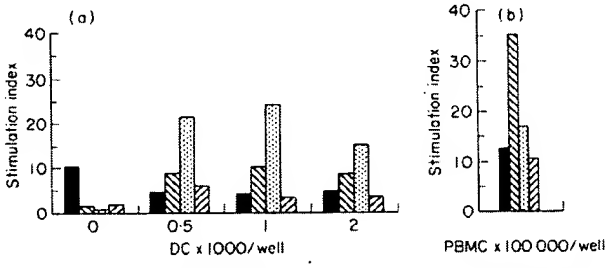

Fig.3. The ability of dendritic cells (DC) to present antigens/mitogen to T lymphocytes. T lymphocytes (10^5) were cocultured in the absence or presence of graded numbers of DC and stimulated with concanavalin A (Con A), soluble *Candida albicans* (CAs), particulate CA (CAp) and purified protein derivative (PPD). The values represent mean stimulation indices (SI) of two to five experiments. Standard deviation of the mean never exceeded 10%. The ct/min values of unstimulated control cultures were < 100. SI values of DC cultured alone were *ca* 1·0 (a). Proliferative responses of unfractionated peripheral blood mononuclear cells (PBMC) to the same stimuli as above (b). ■, Con A (5 μg/ml); ▨, PPD (10 μg/ml); ▨, CAs (1:40); ■, CAp (1:100).

Table 2. Separation of dendritic cells (DC) from the peripheral blood of four HIV-infected individuals

	Cells × 10^6/ml peripheral blood			
Cell fraction	1*	2	3	4
Ficoll-Isopaque-separated	0·58	0·42	1·95	2·58
Non-phagocytic	0·35 (59)†‡	0·33 (80)	0·66 (34)	1·67 (65)
Low density	ND	ND	0·17 (9)	0·1 (4)
Enriched DC	0·02 (3·4)	0·007 (1·6)	0·012 (0·6)	0·008 (0·3)

* Patient number.
† Per cent of starting population.
ND, Not determined.
‡ Numbers in parentheses are percentages.

enrichment method could be used for HIV-infected patient samples. Of four HIV-infected individuals examined, presenting with CDC stage II or III disease, DC numbers comparable to those of peripheral blood DC from non-infected individuals were obtained. Thus, we could not confirm the findings of Macatonia and co-workers [35] on reduced numbers of DC in asymptomatic (CDC stage II) and late stage (CDC stage IV) HIV patients. Macatonia, Knight and co-workers have also reported on the finding that up to 21% of DC are productively infected by HIV as demonstrated by *in situ* RNA/DNA hybridization [35–37]. We could not, however, confirm this finding. It remains possible that in HIV infection blood DC carry few copies of HIV provirus which is beyond the detection limit of the *in situ* hybridization method. In fact, Patterson and co-workers [38] have recently shown that HIV-infected DC express viral DNA in favour of viral RNA, thus suggesting a latent infection. On the other hand, Cameron and co-workers [39] detected only rare HIV DNA-positive DC with the polymerase chain reaction (PCR)-based nucleid acid amplification using conventional isolation methods. Our improved DC enrichment method will now make further studies possible with PCR and viral isolation studies in HIV infection.

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